Production of Isocyclomaltopentaose from Starch
Using Isocyclomaltooligosaccharide Glucanotransferase

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Production of a novel cyclomaltopentaose cyclized by an α-1,6-linkage, [ICG5; cyclo-(→6)-α-D-GlcP-(1→4)-
α-D-GlcP-(1→4)-α-D-GlcP-(1→4)-α-D-GlcP-(1→)], from starch was performed using isocyclomaltooligosaccharide glucanotransferase (IGTase) derived from *Bacillus circulans* AM7. The optimal conditions for ICG5-production from partially hydrolyzed starch were as follows: substrate concentration, 1.0% (w/v); pH, 5.5; temperature, 45°C; reaction time, 24 h; IGTase, 1.0 unit/g-dry solid (DS); isoamylase, 2,500 units/g-DS. The yield of ICG5 reached 25.9% under optimal conditions. ICG5-production was achieved from partially hydrolyzed starch using a crude enzyme preparation containing IGTase. Finally, ICG5 was obtained in a yield of 17.9% (99.3% purity, 2,681 g-DS). A digestive test with a human salivary amylase, an artificial gastric juice, a pancreatic amylase, and small intestinal enzymes showed that ICG5 was an indigestible oligosaccharide.

Key words: cyclomaltopentaose; α-1,6-linkage; *Bacillus circulans*; glucanotransferase; starch

Enzymatic syntheses of various cyclic oligosaccharides have been reported by many researchers. Cyclomaltohexaose (α-CD), one of the most well-known cyclic oligosaccharides, is produced from linear α-1,4-glucans by the intramolecular α-1,4-transglycosylation reaction of a cyclomaltooltriose glucanotransferase (EC 2.4.1.19). The cyclic oligosaccharide has a hydrophobic cavity in the center of the structure. Guest molecules of suitable size can enter the cavity, and the formation of the inclusion complex is used to stabilize labile materials, mask odors, and modify viscosity. Côté and co-workers first reported that a cyclic tetrasaccharide consisting of α-D-glucose, cyclo-(→6)-α-D-GlcP-(1→3)-α-D-GlcP-(1→6)-α-D-GlcP-(1→3)-α-D-GlcP-(1→), was produced from a dextran-like polysaccharide, alternan, by its degrading enzyme. The cyclic oligosaccharide was designated cycloalternan (CA). Recently, we discovered two novel enzymes, 6-α-glucosyltransferase and 3-α-isomaltosyltransferase, in *Bacillus globisporus*, and succeeded in the mass production of this saccharide from starch by the joint reaction of the two enzymes. More recently, we found a new enzymatic system to synthesize a cyclic maltosyl-(1→6)-maltose (CMM), cyclo-(→6)-α-D-GlcP-(1→4)-α-D-GlcP-(1→6)-α-D-GlcP-(1→4)-α-D-GlcP-(1→), from starch. Consequently, we obtained two kinds of cyclic tetrasaccharides by discovering CMM. To distinguish the two kinds of cyclic tetrasaccharides, in this paper we designate the cyclic nigerosyl-(1→6)-nigerose (CNN) instead of CA. CNN and CMM are smaller than the other cyclic glucans and have heterogeneous linkages in their structure. Although the characteristics of CNN and CMM have not yet been totally clarified, potential applications of the saccharides in food, cosmetics, and medicines are anticipated because of its unique structure. Hence cyclic oligosaccharides produced from starch by bacterial enzymes are of great interest from the functional and industrial points of view.

During the course of our screening for microorganisms that produce nonreducing oligosaccharides from amylase, we obtained the bacterial strain *Bacillus circulans* AM7 from soil. Structural analysis showed that the nonreducing oligosaccharides produced by this strain are cyclomaltopentaose cyclized by an α-1,6-linkage. The novel cyclomaltopentaose, designated isocyclomaltopentaose [ICG5, cyclo-(→6)-α-D-GlcP-(1→4)-α-D-GlcP-(1→4)-α-D-GlcP-(1→4)-α-D-GlcP-(1→)], has a unique structure containing an α-1,6-linkage in its molecule. Our previous report showed that the novel glucanotransferase, isocyclomaltooligosaccharide glucanotransferase (IGTase), converted α-1,4-glucan into ICG5 by a one-step reaction (Fig. 1).

In this study, we report on the reaction conditions

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required for the production of ICG5 from starch using IGTase. We also demonstrate ICG5 production using a crude enzyme preparation from *B. circulans* AM7. In addition, we report on some properties of ICG5.

**Materials and Methods**

**Saccharides.** Partially hydrolyzed starch, Pinedex no. 100 (dextrose equivalent 2 to 5), Pinedex no. 1 (dextrose equivalent 8 ± 1), Pinedex no. 4 (dextrose equivalent 19 ± 2), and Pinedex no. 3 (dextrose equivalent 25 ± 2), were purchased from Matsutani Chemical Industry (Itami, Japan). Soluble starch was purchased from Katayama Chemical (Osaka, Japan). Glycogen from corn was purchased from QP (Tokyo). Dextran T-2000 was purchased from GE Healthcare Bio-Sciences (Piscataway, NJ). Maltodextrins, cyclo-dextrins (DP 6, 7, and 8), and amyllose EX-I, whose average degree of polymerization (DP) is 17, were prepared in our laboratory.

**Enzymes.** α-Glucosidase (EC 3.2.1.20) from *Aspergillus niger* was purchased from Amano Enzyme (Nagoya, Japan). Glucoamylase (EC 3.2.1.3) from *Rhizopus* sp. was purchased from the Seikagaku Kogyo (Tokyo). Cyclomaltodextrin glucanotransferase (CGTase; EC 2.4.1.19) from *B. circulans* was prepared in our laboratories. IGTase from *B. circulans* was also prepared in our laboratories.13) The activities of α-glucosidase and glucoamylase were assayed according to the methods recommended by their respective suppliers. The activities of IGTase and CGTase were assayed as reported in previous publications.13,14) Isoamylase (EC 3.2.1.68) from *Pseudomonas amyloleamans* was prepared in our laboratories. The activity of isoamylase was assayed as reported in a previous publication.15)

**Reaction conditions of ICG5 production.** The effects of different types of substrate on the ICG5 production were tested as follows: A reaction mixture (4.0 ml) containing 1.0% (w/v) of each substrate, 50 mM sodium acetate buffer (pH 6.0), 1.0 mM CaCl$_2$, and the 1.0 unit/g-dry solid (DS) of purified IGTase was incubated at 45°C for 24 h. The effects of reaction pH on ICG5 production were tested as follows: A reaction mixture (4.0 ml) containing 1.0% (w/v) of amylase, 50 mM various buffers (sodium acetate, pH 4.5 to 6.0; sodium phosphate, pH 6.0 to 7.5; Tris–HCl, pH 7.5 to 8.0), 1.0 mM CaCl$_2$, and 1.0 unit/g-DS of the enzyme was incubated at 40°C for 24 h. The effects of reaction temperature on ICG5 production were tested as follows: A reaction mixture (4.0 ml) containing 1.0% (w/v) of amylase, 50 mM sodium acetate buffer (pH 6.0), 1.0 mM CaCl$_2$, and 1.0 unit/g-DS of the enzyme was incubated at 45°C for 48 h. The effects of substrate concentrations on ICG5 production were tested as follows: A reaction mixture (4.0 ml) containing 1.0% to 10% (w/v) of Pinedex no. 100, 50 mM sodium acetate buffer (pH 6.0), 1.0 mM CaCl$_2$, and 1.0 unit/g-DS of the enzyme was incubated at 45°C for 48 h. The effects of additional isoamylase on ICG5 production were tested as follows: A reaction mixture (4.0 ml) containing 1.0% (w/v) of Pinedex no. 100, 50 mM sodium acetate buffer (pH 5.5), 1.0 mM CaCl$_2$, 1.0 unit/g-DS of IGTase, and 0 to 2,500 units/g-DS of isoamylase was incubated at 45°C for 48 h.

**Crude enzyme preparation containing IGTase.** *B. circulans* AM7 was cultured in a 30-liter jar fermentor holding a culture medium (20-liter × 4, pH 7.5) composed of 0.25% Pinedex no. 100, 1.2% poly- peptide, 0.1% K$_2$HPO$_4$, 0.06% NaH$_2$PO$_4$·2H$_2$O, 0.05% MgSO$_4$·7H$_2$O, and 0.3% CaCO$_3$. Cultivation was performed at 27°C for 72 h, with the paddle rotation set at 300 rpm and aeration at 8.0-liter/min. The culture broth was then centrifuged for 10,000 × g for 20 min, after which the supernatant was concentrated to 3.1-liter using an ultra filter (API-2013; Asahi Chemical, Tokyo). The resulting crude enzyme mixture contained 16,000 units of IGTase.

**Chromatography using cation-exchange resin.** To purify ICG5 from the reaction mixture, we used a chromatography system composed of 10 columns (12.5 cm i.d. × 160 cm × 10) packed with 225-liter of Diaion UBK530 resin (Mitsubishi Chemical, Tokyo). Chromatography was performed under the following conditions: water as the mobile phase, 45-liter/h (flow rate), 60°C (temperature), and a fraction size of 2 liters.

**Alkaline treatment.** Reducing saccharides were decomposed by alkaline treatment. A 10 M NaOH solution (260 ml) was added into the reaction mixture (12.6 liters) and heated at 100°C for 1 h to keep the pH above 11. The reaction mixture was desalted using 40 liters of

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Fig. 1. Mechanism for the Synthesis of ICG5 from Maltodextrins by the Action of IGTase.

- Glucopyranosyl residue; \( \varnothing \), glucose residue at reducing end; \( \bullet \), position of anomeric carbon; \( \triangle \), catalytic point; solid line, \( \alpha \)-1,4-linkage; broken line, \( \alpha \)-1,6-linkage; \( n = 1, 2, 3 \ldots \)

Cyclization reaction

(\( \alpha \)-1,6-intramolecular transfer reaction)
ion-exchange resins (Diaion SK1B, Amberlite IRA411, and Diaion WA30 in a ratio of 1:2:1; Mitsubishi Chemical and Organo Japan, Tokyo).

**Analysis of ICG5 yield.** After α-glucosidase and glucoamylase treatment of the sample, the yield of ICG5 was analyzed by high performance liquid chromatography (HPLC). An IGTase reaction was terminated by boiling the reaction mixture for 10 min. To hydrolyze residual linear saccharides other than cyclic oligosaccharides, an enzyme solution (0.5 ml) containing α-glucosidase (400 units/g-DS) and glucoamylase (30 units/g-DS) in 50 mM acetate buffer (pH 4.5) was added to the reaction mixture (0.5 ml) and incubated at 50 °C for 60 min. After 10 min of boiling, the solution was filtered using KC prep dura (0.45 μm; Katayama Chemical, Osaka, Japan) and desalted with a micro acilyzer G0 AC-110 (Asahi Chemical, Tokyo). The resulting ICG5 was analyzed by HPLC.

**Stability of ICG5 under heated condition.** Sodium phosphate buffer solution (33 mM, pH 6.0) containing 5.0% (w/v) of ICG5 or α-CD was incubated with an autoclave at 120 °C at 1.2 kg/cm² for 90 min. The amounts of residual saccharides were determined by HPLC.

**Stability of ICG5 under acidic and heated conditions.** An acetic acid solution (100 mM) containing 4.0% (w/v) of ICG5 or α-CD was incubated at 100 °C for 24 h. Hydrochloric acid (pH 2.0) and sodium acetate (pH 3.0 to 6.0) were used to adjust the pH. The amounts of residual saccharides were determined by HPLC.

**Maillard reactivity.** Sodium citrate buffer (100 mM, pH 4.0 to 6.0) and sodium phosphate buffer (100 mM, pH 7.0) containing 5.0% (w/v) of ICG5, α-CD, or maltose and 1.0% (w/v) glycine were incubated at 100 °C for 90 min. Coloring by the Maillard reaction was evaluated from the absorption value at 480 nm.

In vitro digestive test. The digestibility of ICG5, cyclodextrins, and maltose was investigated using a human salivary amylase, an artificial gastric juice, a porcine pancreatic amylase, and rat intestinal enzymes. Digestive tests were performed in a manner identical to the method of Okada et al.16

**HPLC analysis.** HPLC analysis was performed using an LC-10AD pump, an RID-10A refractive index monitor, and a C-R7A data processor (Shimadzu, Kyoto, Japan) equipped with an MCI GEL CK04SS column (10 mm i.d. × 200 mm × 2; Mitsubishi Chemical, Tokyo) at a flow rate of 0.4 ml/min with water as the solvent at 80 °C.

<table>
<thead>
<tr>
<th>Table 1. Effect of Substrate on ICG5 Production</th>
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<tbody>
<tr>
<td>Substrate</td>
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<tr>
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</tr>
<tr>
<td>Maltose</td>
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<tr>
<td>Maltotriose</td>
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<tr>
<td>Maltotetraose</td>
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<tr>
<td>Maltopentaose</td>
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<tr>
<td>Maltohexaose</td>
</tr>
<tr>
<td>Maltoheptaose</td>
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<tr>
<td>Amylose</td>
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<tr>
<td>Pinedex no. 100</td>
</tr>
<tr>
<td>Pinedex no. 1</td>
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<tr>
<td>Pinedex no. 4</td>
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<tr>
<td>Pinedex no. 3</td>
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<tr>
<td>Soluble starch</td>
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<tr>
<td>Glycogen</td>
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</table>

Reaction mixtures (4.0 ml) containing 1.0% of each substrate, 50 mM acetate buffer (pH 6.0), 1 mM CaCl₂, and 1.0 unit/g-DS of IGTase were incubated at 45 °C for 24 h. After α-glucosidase and glucoamylase treatment of the reaction mixture, the yield of ICG5 was measured by HPLC. n.d., not detected.

**Results and Discussion**

**Effect of substrate on ICG5 production**
To select a substrate for the production of ICG5, we examined various starch-related saccharides. As listed Table 1, amylose gave a high ICG5 yield of 27.2%. Second to amylose, soluble starch showed a good ICG5 yield of 26.5%. The yields from partially hydrolyzed starch, Pinedex no. 100, no. 1, no. 4, and no. 3 were 20.1%, 13.9%, 9.7%, and 6.5% respectively. When maltooligosaccharides were used as substrates, the larger the degrees of polymerization (DP), the higher the ICG5 yield. In addition, glycogen having many branches showed a poor ICG5 yield of 14.6%. These results indicate that a substrate having a low dextrose equivalent and no branches is a good substrate to generate ICG5.

**Effect of reaction pH and temperature**
First, the IGTase reaction was performed with amylose at 40 °C and various pHs. As shown in Fig. 2A, the ICG5 yields were nearly the same in pH range 5.0 to 7.9. Second, the reaction was performed at pH 6.0 at various temperatures. As shown in Fig. 2B, a reaction temperature of 45 °C gave a good yield of ICG5. The optimum temperature and pH for the production of ICG5 were considered to be 45 °C and 5.0 to 7.9.

**Effect of substrate concentration**
The effect of substrate concentration on ICG5 yield was investigated using Pinedex no. 100 as a substrate at pH 6.0 at 45 °C. As shown in Fig. 3A, the yield of ICG5 from a substrate concentration of 1.0% was highest, and reached 21.1% after 24 h. The yield of ICG5 decreased remarkably with increases in substrate concentration. The enzyme acted on the substrate to produce ICG5 by an intramolecular α-1,6-glycosyl transfer reaction (cyc-
The enzyme also catalyzed the transfer of part of a linear oligosaccharide to another oligosaccharide by an intermolecular \( /C11 \)-1,4-glycosyl transfer reaction (disproportionation reaction). Because of the high frequency of the disproportionation reaction with increases in the substrate concentration, the cyclization reaction takes place less readily, and the yield of ICG5 decreased. Based on this result, a substrate concentration of 1.0% was considered optimal for ICG5-production.

**Effect of additional isoamylase**

The effect of additional isoamylase on the ICG5 yield was investigated using Pinedex no. 100 as a substrate at 45 °C. The stability of the added isoamylase in pH 6.0 was remarkably low; therefore, the pH was set to 5.5 in this case. As shown in Fig. 3B, the highest yield (25.9%) of ICG5 was obtained when 2,500 units/g-DS of isoamylase was added for 24 h. In the presence of isoamylase, the yield of ICG5 from partially hydrolyzed starch reached 95%; compare this with the yield (27.2%) from amylose. Therefore, Pinedex no. 100, with high solubility in water and good handling, was selected as the substrate for ICG5 production. Thus, the optimal condition for ICG5 production from Pinedex no. 100 was as follows: substrate concentration, 1.0%; pH, 5.5; temperature, 45 °C; reaction time, 24 h; IGTase, 1.0 unit/g-DS; isoamylase, 2,500 units/g-DS.

**Production of ICG5 using a crude enzyme preparation**

A crude enzyme preparation (3.1 liters) from *B. circulans* AM7 contained 16,000 units of IGTase. This enzyme preparation was used as IGTase. A reaction mixture (1,500 liters) containing 1.0% Pinedex no. 100, 10 mM sodium acetate buffer (pH 6.0), 1.0 mM CaCl\(_2\), and 1.0 unit/g-DS of IGTase, and 0 to 2,500 units/g-DS of isoamylase was incubated at 45 °C for 48 h. Isoamylase concentrations: ○, 0; ●, 125 units/g-DS; △, 250 units/g-DS; □, 500 units/g-DS; ×, 2,500 units/g-DS.
g-DS) was added to the mixture and kept pH 5.0 and 50°C for 20 h. After the hydrolysis reaction, the solution was heated to 90°C for 30 min to inactivate the enzyme. The ICG5 yield of the resulting product was 26.1% of the total sugar. After concentration, ICG5 was partially purified by column chromatography with cation-exchange resins. The fractions containing mainly ICG5 were recovered at a yield of 3,566 g-DS. The partially-purified ICG5 (85.0% purity) was treated with CGTase (10 units/g-DS) from *B. circulans* and α-glucosidase (500 units/g-DS) at 40°C for 15 h to hydrolyze the contaminating ICG6 into glucose. As shown in Fig. 4, ICG6 was completely hydrolyzed into glucose by CGTase and α-glucosidase treatment. Furthermore, saccharides whose molecular weight was larger than ICG6 were also hydrolyzed into glucose. We have determined that ICG5 is not hydrolyzed by CGTase from *B. circulans*, though ICG6 is hydrolyzed. In this study, it was found that contaminating saccharides whose molecular weight was larger than ICG6 were also hydrolyzed by CGTase from *B. circulans*. To decompose the contaminating glucose, the reaction mixture was treated with an alkaline. After the alkaline treatment, we successively performed deionization, filtration, and concentration. Finally, we obtained ICG5 (99.3% purity) at a yield of 2,681 g-DS.

Some properties of ICG5

Some properties of ICG5 were investigated mainly in order to evaluate its usefulness in processed foods. First, its stability under heated condition was examined. ICG5 was undegraded at 120°C for 90 min as well as α-CD (no degradation ratio: ICG5 100%, α-CD 100%). Second, its stability under acidic and heated conditions was investigated. ICG5 was stable at pH 5.0 and above, but easily degraded at pH 3.0 and below (Fig. 5A). Third, the Maillard reaction was evaluated. Figure 5B shows the results of a test conducted on heat coloring in the presence of amino acid. ICG5 caused no Maillard reaction. Finally, an *in vitro* digestive test was carried out. As shown in Table 2, no hydrolysis of ICG5 was observed by human salivary amylase, artificial gastric juice, or porcine pancreatic amylase. Furthermore, the hydrolysis was negligible by rat intestinal enzymes. These results indicate that ICG5 is an indigestible oligosaccharide. The α-CD of indigestible oligosaccharides...
ride is classified as dietary fiber; therefore, ICG5 is expected to be utilized as a new food material having a function like dietary fiber.

Conclusions
We produced a novel saccharide, ICG5, from starch using a novel enzyme, IGTase, derived from a Bacillus strain. We finally obtained 2,681 g of ICG5 from 15,000 g of a partially hydrolyzed starch. Some properties were examined using the produced ICG5. ICG5 showed no Maillard reaction at a high temperature. In addition, it was not hydrolyzed by human salivary amylase, artificial gastric juice, pancreatic amylase, or small intestinal enzymes. These results suggest that ICG5 should be utilized as a new food material having a function like dietary fiber. We are progressing in the technology for producing ICG5 on an industrial scale. In the future, we expect that useful functions of ICG5, different from those of CDs, will be developed.

References
12) Watanabe, H., Nishimoto, T., Sonoda, T., Kubota, M., Chena, H., and Fukuda, S., An enzymatically produced cyclomaltopentasaccharide cyclized from amylase by an α-(1→6)-linkage, cyclo-[α-1,6-glucose)-1,4-α-1,6-glucose)-1,4-α-1,6-glucose)-1,4-α-1,6-glucose)-1,4-α-1,6-glucose), from starch. Carbohydr. Res., 340, 1469–1474 (2005).

Table 2. In Vitro Digestibility of ICG5

<table>
<thead>
<tr>
<th>Residual rate (%)</th>
<th>ICG5</th>
<th>α-CD</th>
<th>β-CD</th>
<th>γ-CD</th>
<th>Maltose</th>
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<tbody>
<tr>
<td>Human salivary α-amylase</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Artificial gastric juice</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Pancreatic amylase</td>
<td>100</td>
<td>100</td>
<td>99.8</td>
<td>94.7</td>
<td>100</td>
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<tr>
<td>Rat small intestine enzymes</td>
<td>99.8</td>
<td>99.7</td>
<td>98.7</td>
<td>42.5</td>
<td>12.8</td>
</tr>
</tbody>
</table>

Each value is the average of three replicate measurements.